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Developmentally Regulated *Xist* Promoter Switch Mediates Initiation of X Inactivation

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Summary

Developmental regulation of the mouse *Xist* gene at the onset of X chromosome inactivation is mediated by RNA stabilization. Here, we show that alternate promoter usage gives rise to distinct stable and unstable RNA isoforms. Unstable *Xist* transcript initiates at a novel upstream promoter, whereas stable *Xist* RNA is transcribed from the previously identified promoter and from a novel downstream promoter. Analysis of cells undergoing X inactivation indicates that a developmentally regulated promoter switch mediates stabilization and accumulation of *Xist* RNA on the inactive X chromosome.

Introduction

X chromosome inactivation (X inactivation) provides the mechanism of dosage compensation for X-linked genes in XX female mammals relative to XY males (reviewed in Heard et al., 1997). Both X chromosomes are active in early preimplantation XX embryos, and transcriptional silencing of a single X chromosome proceeds as cells differentiate from totipotent embryonic lineages (Monk and Harper, 1979). Studies in mouse have shown that X inactivation first occurs in the extraembryonic trophectoderm and primitive endoderm lineages at the blastocyst stage. In these cells X inactivation is paternally imprinted (i.e., the paternally derived X chromosome [Xp] is preferentially inactivated [Takagi and Sasaki, 1975]). X inactivation in the embryo proper occurs at the time of gastrulation in epiblast cells of postimplantation embryos (McMahon et al., 1983). In this case, initiation of X inactivation is random, with an equal probability of either the maternally derived X chromosome (Xm) or Xp being inactivated in a given cell. The inactive state is heritably maintained in all cell divisions following initiation of either random or imprinted X inactivation.

Developmental regulation of X inactivation requires a *cis*-acting master switch locus termed the X inactivation center (Xic). The Xic is involved in the initiation process whereby cells determine appropriate X inactivation patterns, and it is absolutely required in *cis* for propagation of the inactive state along the chromosome (reviewed in Rastan and Brown, 1990). Initiation of random X inactivation involves determining how many (counting) and which (choosing) X chromosome to inactivate. It has been suggested that this is achieved by cells blocking a single Xic and thus marking that chromosome as the active X chromosome. X inactivation then proceeds in *cis* from unblocked Xics at the onset of cellular differentiation (Rastan, 1983).

The X inactive specific transcript (*Xist*) gene, originally identified as a candidate for the Xic (Brown et al., 1991a, 1991b; Borsani et al., 1991; Brockdorff et al., 1991), produces a large RNA with no apparent protein coding potential (Brockdorff et al., 1992; Brown et al., 1992). *Xist* RNA "coats" the inactive X chromosome domain in the interphase nucleus, suggesting it could provide the primary signal for in *cis* propagation of X inactivation (Brown et al., 1992; Clemson et al., 1996). The earliest *Xist* expression in mouse embryos is detectable at the 4- to 8-cell stage (Kay et al., 1993). Expression is exclusively from the paternal allele, correlating with the paternally imprinted X inactivation that occurs subsequently in trophectoderm and primitive endoderm lineages. Biallelic up-regulation of *Xist* RNA occurs immediately prior to gastrulation (i.e., at the onset of random X inactivation in epiblast cells [Kay et al., 1993] and also in differentiating XX embryonic stem [ES] cells, an in vitro model for random X inactivation [Kay et al., 1993]).

A requirement for *Xist* in *cis* for propagation of X inactivation has been demonstrated by targeted deletion of transcribed regions (Penny et al., 1996; Marahrens et al., 1997). Importantly, the counting function of the Xic was unaffected in these experiments. A more recent gene targeting experiment indicates that sequences distal to *Xist* are important in counting (Clerc and Avner, 1998). Analysis of XY ES cells bearing a 450 kb *Xist* YAC transgene demonstrated that *Xist* is sufficient for X inactivation (Lee et al., 1996) and that transgenic loci recapitulate both counting and propagation functions (Lee et al., 1996; Lee and Jaenisch, 1997). This was subsequently shown using a much smaller 35 kb transgenic *Xist* construct that encompasses *Xist*, 9 kb of upstream sequence, and 6 kb of downstream sequence (Herzing et al., 1997).

We and others have recently shown that developmental up-regulation of *Xist* on the inactive X chromosome (Xi) allele is attributable to increased RNA stability (Panning et al., 1997; Sheardown et al., 1997a). Prior to random X inactivation unstable RNA is transcribed from active X chromosome (Xa) *Xist* alleles in epiblast cells and in undifferentiated ES cells. As cells differentiate,

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Xi transcript is stabilized but Xa alleles continue to transcribe unstable RNA for a short period. Xa transcription is subsequently silenced (Panning et al., 1997; Sheardown et al., 1997a). In this study we show that the developmentally regulated changes in stability of *Xist* RNA are attributable to a promoter switch giving rise to stable and unstable RNA isoforms. Stable *Xist* RNA initiates from the previously published promoter or from a novel promoter located 1.5 kb further downstream. In contrast, unstable *Xist* RNA initiates at a second novel promoter located 6.5 kb upstream. Our observations demonstrate that promoter switching regulates *Xist* RNA stability at the onset of random and imprinted X inactivation.

Results

Multiple Promoters Control *Xist* Gene Expression

A single promoter has been identified in previous studies on the mouse *Xist* gene (Brockdorff et al., 1992; Sheardown et al., 1997b) and also the human gene (Hendrich et al., 1993, 1997). Here we refer to this promoter as P_1. Preliminary evidence for additional promoters came from analysis of *Xist* steady-state RNA levels. First, we obtained evidence that a novel promoter, designated P_2, is located downstream of P_1 and represents the major promoter utilized in XX somatic cells. As shown in Figure 1A, hybridization of probe mx1 to XX somatic cell RNA gives markedly stronger signal than probe mx2. This difference was observed using other downstream *Xist* probes instead of mx1. Quantitative analysis based on independent experiments indicates that 70%–90% of total XX somatic cell RNA initiates from P_2 (Figure 1B).

The experiment shown in Figure 1A also provided initial evidence for a novel upstream promoter, designated P_0, that is specific for ES cells. As expected, probe mx3, located immediately upstream of P_1, does not hybridize significantly to XX somatic cell RNA. However, hybridization of mx3 to XY and XX ES cell RNA occurs at a similar level to that seen with probes mx1 and mx2. This suggested that unstable ES cell *Xist* transcripts initiate upstream of P_1. Quantitative analysis indicates that all three probes hybridize to XY ES cell RNA at a similar level (Figure 1C). Levels in XX ES cells are generally higher, as unstable transcript is expressed from two alleles (Sheardown et al., 1997a). Levels detected with mx1 in XX ES cells were more variable, possibly reflecting the fact that a small proportion of cells spontaneously differentiate and express stable P_1 and P_2 transcripts.

ES cell specificity of upstream transcripts was confirmed by RT-PCR analysis as illustrated in Figure 2. The CJ1/CJ2 primer pair in *Xist* exon 1 amplifies product from ES cell and XX somatic cell cDNA, whereas CJ3/CJ4 and CJ5/CJ6 primer pairs, located 1.3 and 5 kb upstream of P_1 respectively, only amplify product from ES cell cDNA. Finally, primer pair CJ7/CJ8 located 8 kb upstream failed to amplify product from any of the samples. This indicates that P_0 lies between 5 and 8 kb upstream of P_1.

Analysis of the Major Somatic Promoter P_2

Quantitative steady-state RNA analysis narrowed down the location of P_2 to a region 1–2 kb downstream of P_1 (Figure 1A). We used nuclease protection analysis to fine map the actual site of initiation of transcription to position +1503. Figure 3A illustrates an experiment using mung bean nuclease and the antisense protection probe RP2. The protected band (P_2) was readily detectable using 10 μg XX somatic cell RNA (lanes 5 and 6) and was absent in negative controls (lanes 2 and 4). In addition to this major band we observed a weaker full-length protected product (FL) corresponding to transcripts originating from P_1. The relative intensities of the FL and P_2 bands is consistent with the previously determined proportion of transcripts originating from P_1 and P_2 (Figure 1B). Identical protected products were seen in experiments using S1 nuclease (data not shown). We did not detect protected products using 10 μg total RNA from XY ES cells (lane 3), presumably because of the very low abundance of unstable *Xist* RNA in ES cells.

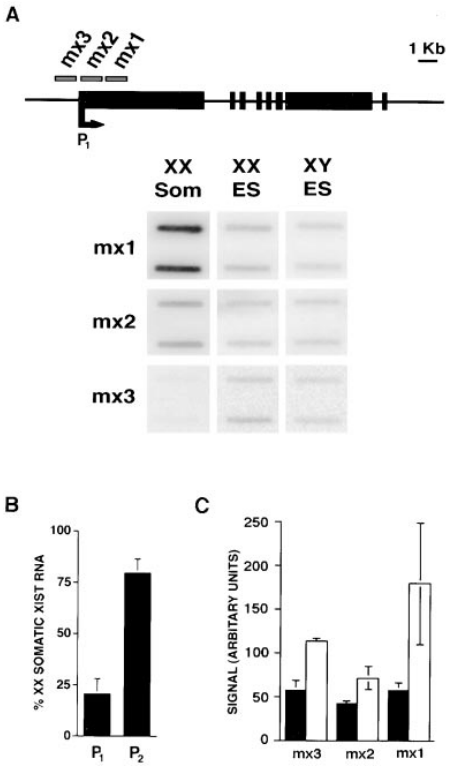

Figure 1. Multiple Promoters Control *Xist* RNA Expression

(A) Hybridization of probes mx1–mx3 to RNA slot blots with duplicate loadings of 10 μg of RNA from XX somatic cells, XX ES cells, and XY ES cells. The location of probes relative to the previously reported promoter P_1 is indicated in the schematic above. Results indicate the presence of a novel downstream promoter utilized in XX somatic cells and a novel upstream promoter utilized in ES cells.

(B) Quantification of RNA slot blot data for probes mx1 and mx2 in XX somatic cells, illustrating that the majority of transcripts initiate from the downstream promoter P_2. Values shown indicate percent of total somatic RNA initiating from P_1 or from P_2.

(C) Quantification of RNA slot blot data for probes mx1–mx3 in XY ES cells (filled bars) and XX ES cells (open bars). Similar levels of signal are seen with all three probes. Values shown in (B) and (C) represent the mean of at least three independent hybridizations in which samples were subtracted for background and normalized to signal for 28s rRNA. To directly compare different probes, data were normalized to *Xist* cosmid DNA control sample.

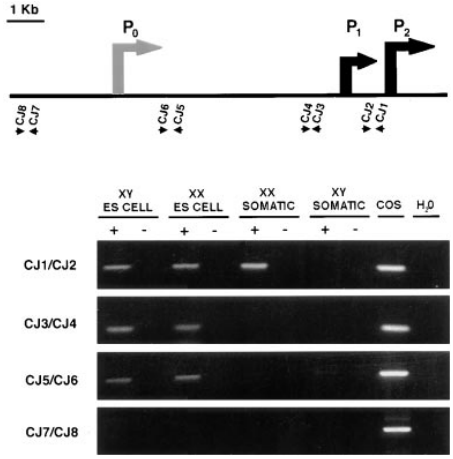

Figure 2. RT-PCR Analysis Demonstrating ES Cell Specificity of Upstream Transcripts

The location of primers CJ1–CJ8 is indicated relative to *Xist* promoters in the schematic. The provisional mapping of promoter P_0 is indicated with the shaded arrow. RT-PCR was carried out on cDNA reactions (+ and −RT) prepared from XY and XX ES cells and from XY and XX somatic cells. Positive control template was *Xist* cosmid DNA (cos). Negative control lane has no template (H_20).

Protection experiments using RP_1 and RP_3 showed full-length protected product as a single band for XX somatic cell RNA (data not shown), suggesting that transcripts originate from the single mapped site within the P_2 region. There is no obvious TATA box consensus in close proximity to the P_2 start site, a feature also noted for promoter P_1 (Brockdorff et al., 1992; Sheardown et al., 1997b).

The difference in steady-state levels of P_1 versus P_2 transcripts could be attributable to different transcription rates, differences in relative stability, or a combination of the two. To analyze this, we compared P_1 and P_2 transcript stability by quantitating RNA levels in XX somatic cells treated for various periods with the transcriptional inhibitor ActinomycinD. The results, illustrated in Figure 3B, show that there is no detectable difference using a probe specific for P_1 transcripts and a probe that detects both P_1 and P_2 transcripts. Calculated $t_{1/2}$ values are similar to those obtained for somatic RNA in previous experiments (Sheardown et al., 1997a). Thus, predominance of P_2 transcripts is attributable to higher transcriptional activity of P_2 relative to P_1. While our data indicate that P_1 and P_2 transcripts have similar properties, we cannot rule out the possibility that they have distinct functions in XX somatic cells.

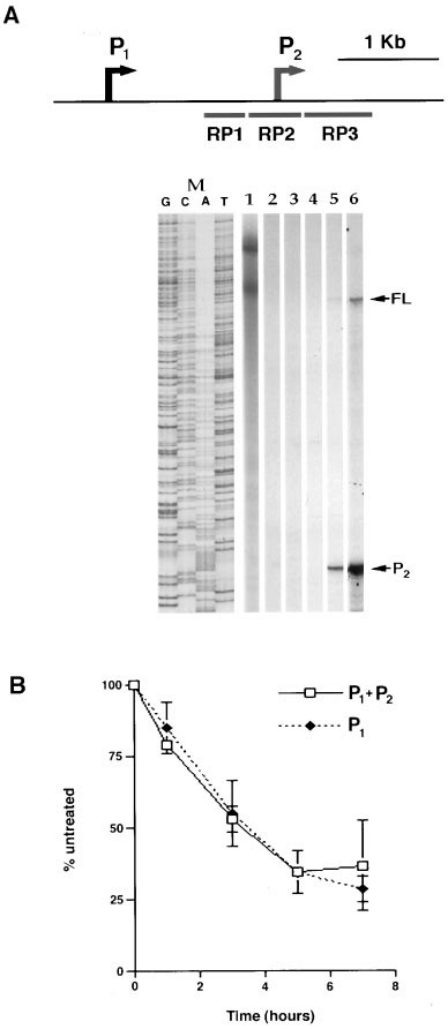

Figure 3. Analysis of Major Somatic Promoter P_2

(A) Detection of P_2 site of initiation of transcription by nuclease protection analysis. The position of antisense probes RP1–RP3 is indicated in the schematic. Mung bean nuclease protection assay using radiolabeled RP2 probe is shown below. Size markers (M) are an RP2 sequencing ladder. Control lanes are undigested probe (lane 1) and nuclease digested probe (lane 2) after hybridization to yeast RNA. Samples are XY ES cell RNA (lane 3), XY somatic cell RNA (lane 4), XX fibroblast cell line RNA (lane 5), and XX somatic tissue RNA (lane 6). A major protected band (P_2) is seen for XX somatic RNA (lanes 5 and 6), mapping the transcription start site to +1503 bp. A weaker full-length protected band (FL) that corresponds to transcripts initiated at P_1 is also seen.

(B) Stability of P_1 *Xist* transcripts (mx2 probe) relative to P_1 + P_2 transcripts (w7d probe) was determined by quantitation of hybridization to RNA from XX somatic cells treated with ActinomycinD for up to 7 hr. Signal was normalized to 28s rRNA and is expressed as a percentage of that seen in untreated cells.

RNA FISH Analysis of P_0 Transcription

Previous studies have shown that unstable *Xist* RNA, as seen in ES cells, appears as a fine punctate nuclear signal (Panning and Jaenisch, 1996; Sheardown et al., 1997a). This contrasts with stable *Xist* RNA in XX somatic cells, which is seen as a large nuclear signal covering the inactive X chromosome territory (Brown et al., 1992; Clemson et al., 1996). To further analyze transcription from promoter P_0, we carried out dual-color RNA FISH using mx7, a probe that spans approximately 7 kb of exon 1 in conjunction with mx8, spanning 5 kb of sequence upstream of P_1 (Figure 4). Probes mx7 (red) and mx8 (green) both detected the fine punctate signal corresponding to unstable *Xist* RNA in undifferentiated XY and XX ES cells (Figures 4A and 4B, respectively). In counting experiments a single colocalizing signal was seen in 96% of XY ES cells (n = 317) and from both alleles in 81% of XX ES cells (n = 161). Similar results

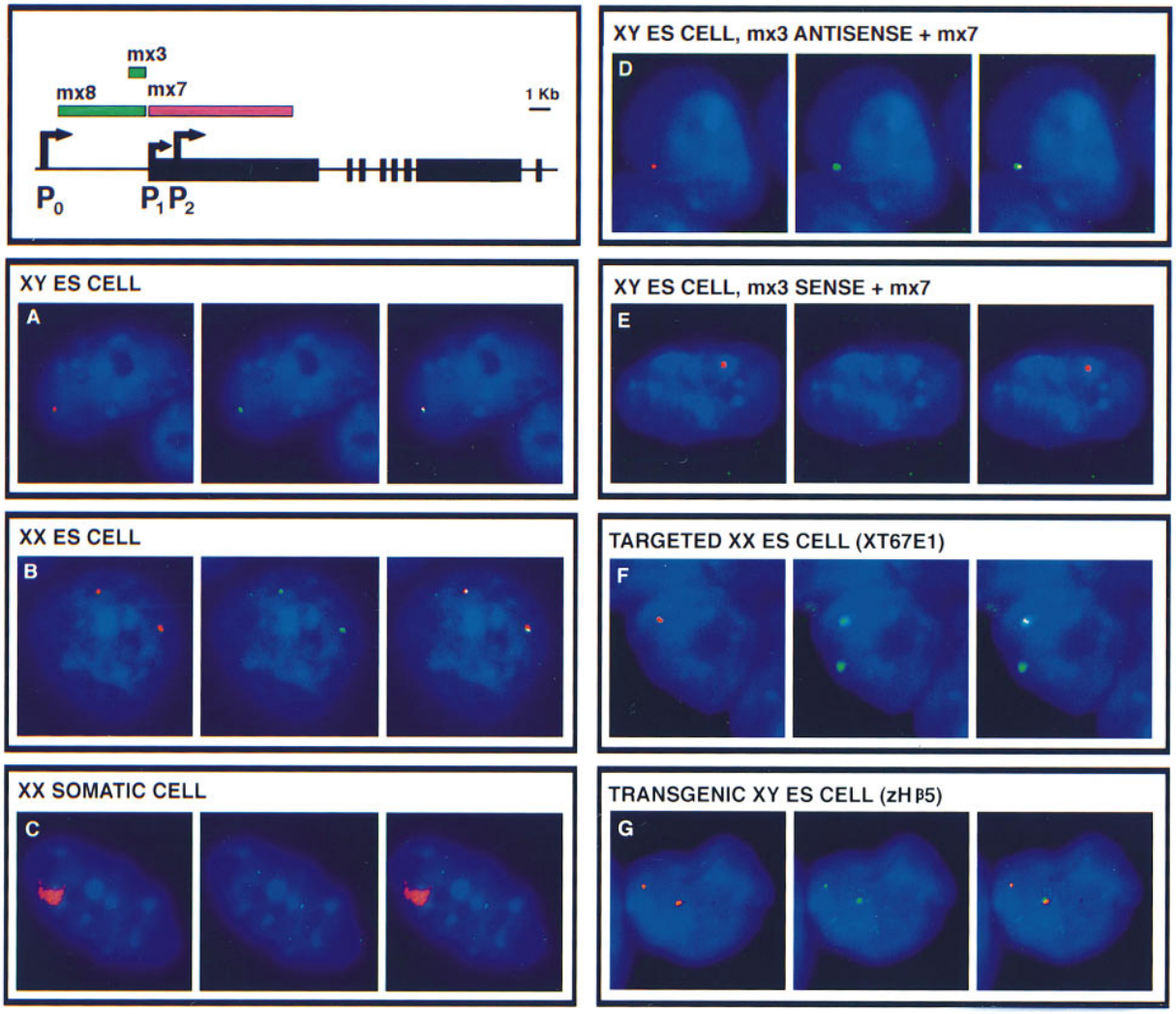

Figure 4. RNA FISH Analysis of Promoter P_0

RNA FISH was carried out using probe mx7 located in exon 1 in conjunction either with upstream probe mx8 (A–C, F, and G), mx3 antisense probe (D), or mx3 sense probe (E). The location of probes is shown in the schematic. Color coding indicates FITC (green) or TRITC (red) detection. Cells were counterstained with DAPI. Sets of three panels show left to right TRITC signal, FITC signal, and merged image. Shown are representative examples of (A) XY ES cells, (B) XX ES cells, (C) XX somatic cells, (D) XY ES cells hybridized with mx7 and antisense mx3 probes, (E) XY ES cells hybridized with mx7 and sense mx3 probes, (F) XT67E1 targeted XX ES cells, and (G) zHβ5 *Xist* transgenic XY ES cells.

were obtained with other XY and XX ES cell lines (data not shown). In contrast to this, accumulated stable transcript in XX somatic cells was detected only with the mx7 probe located in exon 1 (Figure 4C). In no case did we observe colocalizing signal with the upstream probe ($n = 843$).

Directionality of P_0 transcripts in the upstream region was confirmed using strand-specific probes generated by linear PCR from mx3 (corresponding to 1 kb immediately upstream of P_1). Dual-color FISH analysis was carried out using a strand-specific probe (green) in conjunction with double-stranded mx7 probe (red). In XY ES cells, we observed colocalizing punctate signal with the antisense probe (Figure 4D) but not the sense probe (Figure 4E). Identical results were seen for strand-specific probes located 3–4 kb upstream and on both alleles in XX ES cells (data not shown).

We next analyzed XT67E1 cells, an XX ES cell line in which both P_1 and P_2 and most of exon 1 of the *Xist* gene are deleted on a single allele (Penny et al., 1996). As expected, only a single punctate signal is detected using mx7 probe (which spans the deleted region), but, interestingly, punctate signal from both alleles is seen with mx8 (Figure 4F). This indicates that the activity of the P_0 promoter in ES cells is independent of P_1 and P_2.

Finally, we analyzed XY ES cell lines zHβ5 and zHβ10, which carry two and eight tandem copies, respectively, of an ectopically integrated 35 kb *Xist* cosmid (Herzing et al., 1997). The transgene includes the *Xist* gene, 9 kb of upstream sequence, and 6 kb of downstream sequence. Previous studies have shown that these cell lines register the transgene as a countable Xic and up-regulate either endogenous or ectopic *Xist* upon differentiation (Herzing et al., 1997). We observed colocalizing punctate

signal from two alleles in both zHβ5 and zHβ10 ES cells. A stronger signal was seen on one allele, consistent with expression from multiple copies of the transgene locus (Figure 4G). Detection of punctate signal from ectopic cosmid loci using mx8 probe confirms that P_0 lies within 9 kb upstream of P_1.

Molecular Analysis of Promoter P_0

The site of initiation of transcription from P_0 was narrowed down to within a 135 bp region 6590–6725 bp upstream of P_1 using RT-PCR analysis (Figure 5A). Products were readily amplified from XX and XY ES cell cDNA using primer pairs CJ9/CJ10 and CJ11/CJ12 (6.6 kb upstream of P_1). However, the primer combination CJ12/CJ13, where CJ13 is located 135 bp upstream of CJ11, did not amplify product. Other primer pairs located upstream of this point, for example CJ14/CJ15, also failed to amplify product. Examination of sequence in this region revealed no consensus TATA box close to the P_0 transcription start site.

To determine whether P_0 is the major site of initiation of unstable *Xist* RNA, we carried out quantitative steady-state analysis on XY ES cell RNA using additional single-copy probes derived from the region upstream of P_1. As shown in Figure 5B, ES cell transcript is detected with probes mx4 and mx5 (3 kb and 5.5 kb upstream, respectively) but not with mx6 (7 kb upstream). Data for probes mx1 to mx3 from Figure 1C are shown for comparative purposes. The strength of hybridization of probes mx1 to mx5 is similar in all cases, consistent with initiation of a contiguous unstable transcript from the upstream promoter P_0.

Figure 5C illustrates GpC/CpG sequence composition analysis of the region spanning all three promoters. Interestingly, there is a lack of CpG suppression characteristic of CpG islands immediately upstream of P_0. CpG-rich regions are also associated with the P_1 and P_2 promoters. Previous studies have demonstrated that CpG residues in the P_1 and P_2 regions are hypomethylated only on the expressed *Xist* allele in XX somatic cells (Norris et al., 1994).

Developmentally Regulated *Xist* Promoter Switching

Recent studies have demonstrated rapid stabilization of *Xist* RNA on the Xi allele at the onset of random X inactivation. The Xa allele continues to produce unstable transcript for a short period before being fully silenced (Panning et al., 1997; Sheardown et al., 1997a). To investigate *Xist* promoter usage at this critical stage, we used dual-color RNA FISH with probes mx7 and mx8 (Figure 6). Both unstable Xa and stable Xi transcripts are present in the majority of XX ES cells at early stages of differentiation (1–3 days) (Sheardown et al., 1997a). In these cells we always observed accumulated Xi RNA signal with the mx7 probe alone and unstable Xa RNA with both mx7 and mx8 probes (Figure 6A). The mx8 probe was never seen to hybridize to accumulating Xi RNA. The same pattern was seen in cells from 6.5 days post coitum (dpc) XX embryos (i.e., immediately after the onset of random X inactivation in vivo [Figure 6B]). Thus, accumulation of stable *Xist* RNA in *cis* at the onset of X inactivation correlates absolutely with a switch from P_0

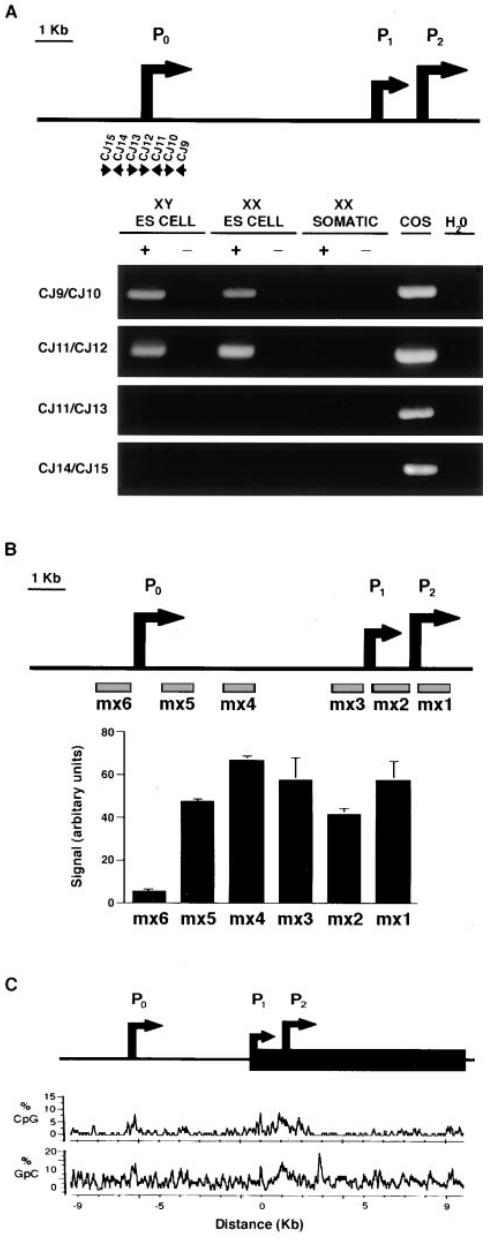

Figure 5. Molecular Analysis of P_0

(A) Fine mapping of P_0 by RT-PCR analysis. Location of primers is indicated in the schematic. RT-PCR analysis demonstrates that P_0 lies within a 135 bp interval between primer CJ12 and CJ13 (6590–6725 bp upstream of P_1). Controls were cDNA prepared with (+) and without (−) RT, positive cosmid DNA control template (cos), and negative no template control (H_20).

(B) Quantitation of steady-state levels of P_0 transcripts in XY ES cells. The location of probes mx1–mx6 is indicated on the schematic. Data for mx1–mx3 is from Figure 1 and is shown for comparative purposes. RNA slot blot data were quantitated as described in Figure 1. Similar levels of signal are seen with all probes except mx6, which lies immediately upstream of P_0.

(C) Sequence composition analysis illustrating CpG and GpC dinucleotide frequencies in an 18 kb region spanning promoters P_0, P_1, and P_2 (indicated in the schematic). CpG-rich regions are associated with all three promoters.

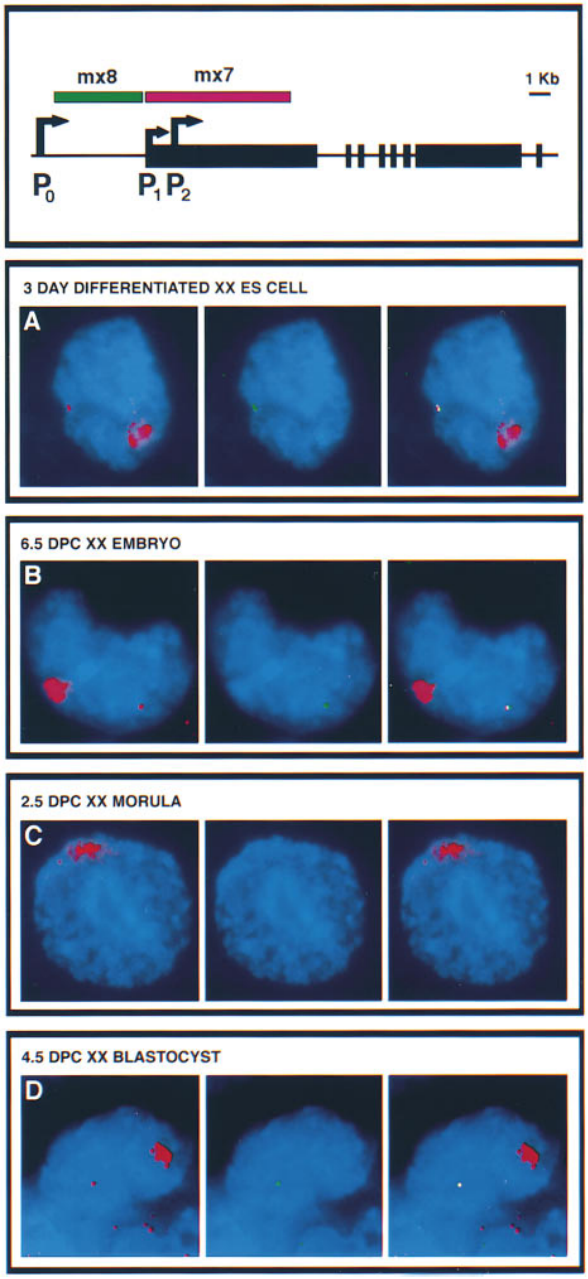

Figure 6. RNA FISH Analysis Illustrating Promoter Usage at the Onset of X Inactivation

The schematic illustrates probe location and is color coded to indicate TRITC (red) or FITC (green) detection. Cells were counterstained with DAPI. Panels show left to right TRITC signal, FITC signal, and merged image. Shown are representative examples of cells from (A) 3-day differentiated XX ES cell culture, (B) 6.5 dpc XX embryo, (C) 2.5 dpc XX morulae, and (D) 4.5 dpc XX blastocyst.

to P_1/P_2 transcription. Later stage differentiated XX ES cells, and also later stage XX embryos (>8.5 dpc), exhibit the same pattern as XX somatic cells (i.e., accumulated Xi signal was detected with mx7 only, with no signal on the Xa allele [data not shown]).

Correlation of RNA stability and promoter usage was also found for imprinted *Xist* expression. Previously we showed that *Xist* RNA transcribed from Xp in early preimplantation mouse embryos (<3.5 dpc) accumulates in *cis*, prior to X inactivation. Signal from Xm is rarely detected until the blastocyst stage and is always punctate (Sheardown et al., 1997a). Examining early preimplantation stages, we found that the accumulating Xp RNA signal is only detected with the mx7 probe (Figure 6C). In blastocysts, where punctate Xm signal is observed, it is detectable with both mx7 and mx8 probes (Figure 6D). These results suggest that imprinted *Xist* expression is attributable to constitutive P_1/P_2 activity on the Xp allele. A switch to P_0 transcription on both the Xp and silent Xm alleles presumably underlies erasure of the imprint required prior to the onset of random X inactivation in the epiblast.

Discussion

In this study we show that developmental regulation of *Xist* expression at the onset of X inactivation is mediated by alternative promoter usage. Prior to random X inactivation, *Xist* is transcribed from a novel upstream promoter P_0 in ES cells and in cells of the epiblast in the developing embryo. P_0 transcripts are highly unstable and do not accumulate in *cis*. A switch to the downstream promoters P_1 and P_2 occurs on the Xi allele at the onset of X inactivation. P_1/P_2 transcripts are stable and accumulate in *cis*. The Xa allele continues to express unstable transcript from P_0 for a short period and is ultimately fully silenced at the transcriptional level. Imprinted X inactivation in trophectoderm and primitive endoderm appears to result from predetermined P_1/P_2 transcription on the Xp allele and absence of Xm transcription.

Correlation of Promoter Usage and Transcript Stability

Alternative promoter usage is a common mechanism in the regulation of gene expression. Variant transcripts can differ in rate of transcription, tissue or developmental stage specificity, coding potential, cellular localization, or mRNA stability (reviewed in Ayoubi and Van de Ven, 1996). Our data indicate that *Xist* promoter usage determines RNA stability. Specifically, unstable and stable *Xist* transcripts appear to represent distinct RNA isoforms that initiate at promoter P_0 or promoters P_1/P_2, respectively. The coexistence of stable and unstable RNA molecules within individual cells at the initiation of random X inactivation suggests that stability and *cis* accumulation are not conferred by cell type–specific factors but are inherent to the RNA isoform. Consistent with this, the accumulating RNA seen in cells of early preimplantation embryos is transcribed from P_1/P_2 but not from P_0.

The hypothesis that *Xist* RNA isoforms determine stability leads to the prediction that the unstable *Xist* transcripts observed in ES cells initiate exclusively from P_0 and not from P_1/P_2. In support of this, analysis of steady-state RNA levels in XY ES cells indicates that P_0 transcripts are contiguous throughout their length. In addition, primer pairs flanking both P_1 and P_2 readily amplify RT-PCR product from ES cell cDNA (data not shown).

Nevertheless, these results do not completely rule out that a low level of unstable transcripts could initiate from P_1 or P_2. To further test the hypothesis that stability is determined by *Xist* RNA isoforms independent of cell background, it will be interesting to analyze ectopic *Xist* constructs driven by a heterologous constitutive promoter. The model predicts that ectopic transcripts initiated at P_1 or P_2 will be stable and accumulate in *cis* both in somatic cells and in undifferentiated ES cells. In contrast, transcripts initiated at P_0 should be unstable both in ES cells and somatic cells.

A possible mechanism for regulation of differential stability is that the 5′ end of the P_0 transcript includes sequences that target rapid degradation by ribonucleases. An alternative, though not mutually exclusive hypothesis is that the 5′ end of the P_0 transcript targets specific RNA transport factors. Diversion of *Xist* RNA away from the site of synthesis, perhaps out of the nucleus, may result in rapid degradation. There are numerous examples of sequence elements responsible for rapid degradation of mRNA, for example in the 5′UTR of the *c-myc* oncogene (reviewed in Atwater et al., 1990; Marcu et al., 1992). Significantly, it has been reported that alternate promoter usage in the human *HOX-5.1* gene gives rise to mRNA isoforms that differ in their 5′UTR and also in their stability (Cianetti et al., 1990).

Control of X Inactivation

Previous studies have shown that there is a developmentally regulated region-specific demethylation over the P_1/P_2 region in the male germline (Norris et al., 1994). It has been reported that this region is methylated in oocytes (Ariel et al., 1995; Zuccotti and Monk, 1995). Taken together these findings led to the suggestion that methylation patterns underlie imprinted *Xist* expression in early preimplantation embryos. Erasure of the imprint prior to the onset of random X inactivation in epiblast cells has been suggested to occur as a result of global demethylation at the blastocyst stage (Norris et al., 1994). Our results indicate that imprinted *Xist* expression occurs as a result of activation of P_1/P_2 on the Xp allele, and that erasure prior to the onset of random X inactivation involves activation of P_0 on both alleles. In light of this, it will be interesting to determine the methylation status of the P_0 region on Xp and Xm both in the developing germlines and before and after erasure of the imprint in early preimplantation embryos.

Initiation of random X inactivation (counting and choice) occurs according to an n-1 rule in which diploid cells are proposed to "block" a single Xic at the onset of X inactivation. As cells differentiate, X inactivation proceeds in *cis* from unblocked Xics (Rastan, 1983). In the context of our data, the developmentally regulated switch from P_0 to P_1/P_2 transcription represents the default situation occuring on n-1 alleles. Blocking would thus equate to repressing P_1/P_2 activity on a single allele. One possible model is that the promoter switch involves mutually exclusive usage of shared enhancer/repressor(s) elements, such that maintenance of P_0 transcription precludes transcription from P_1/P_2. This resembles the enhancer sharing mechanism proposed to explain reciprocal imprinting of the *H19* and *Igf2* loci (Leighton et al., 1995).

Our data suggest that there may be a significant correlation between the expression of unstable RNA from P_0 and the ability of an Xic to be registered by the counting mechanism. The *Xist* deletion in XT67E1 XX ES cells results in failure to X inactivate in *cis*. The counting function of the Xic, however, is unaffected (Penny et al., 1996). Since the deletion extends into the minimal promoter of P_1, it was originally assumed that the deleted allele was null for *Xist*. Consistent with this we detected no *Xist* RNA expression immediately downstream of the deletion (Penny et al., 1996). Data presented here, however, reveal that both alleles express unstable transcript from P_0. Similarly, a second *Xist* deletion that retains the counting function but fails to inactivate in *cis* was found to express unstable *Xist* RNA, presumably from P_0, from the deleted allele in undifferentiated cells (Marahrens et al., 1998). Additionally, we observed that the *Xist* cosmid transgene loci in lines zHβ5 and zHβ10, which are registered by the counting mechanism, express unstable P_0 transcript. Similarly, both counting and P_0 expression have been observed in vivo with an *Xist* transgene insertion on the Y chromosome (M. Ager, personal communication).

A recent study has shown that deletion of a 65 kb region distal to *Xist* results in an allele that is apparently not registered by the counting mechanism (Clerc and Avner, 1998). The chromosome bearing this allele undergoes X inactivation in *cis* by default; the *Xist* gene remains intact and transcribes stable accumulating RNA, presumably from P_1/P_2, when cells differentiate. Interestingly, little or no unstable transcript was detected from the deleted allele in undifferentiated cells. Thus, in this study, P_0 transcription again correlates with the ability of an *Xist* allele to be registered by the counting mechanism. The data also suggest that regulatory elements distal to *Xist* are important in P_0 transcription.

Based on the above observations we suggest that transcription from P_0 is required for an *Xist* allele to be registered by the counting mechanism and that transcription from P_1/P_2 is required for X inactivation in *cis*. This hypothesis leads to certain testable predictions. First, deletion of P_0 would result in an allele that cannot be counted and inactivates by default. Second, deletion of P_0 together with P_1/P_2 would result in an allele that cannot be counted and cannot inactivate in *cis*. This would be equivalent to the situation in the HD2 XX ES cell line in which the entire Xic region is deleted on one allele (Rastan and Robertson, 1985).

Experimental Procedures

Cell Lines

ES cell lines used in this study were the PGK12.1 XX ES cell line (Norris et al. 1994), Efc-1 XY ES cells (provided by A.G. Smith), targeted XT67E1 XX ES cells (PGK12.1 deleted for *Xist* exon 1, Penny et al., 1996), and *Xist* transgenic XY ES lines zHβ5 and zHβ10 carrying two and eight tandem copies, respectively, of an *Xist* cosmid transgene (Herzing et al., 1997; kindly provided by A. Ashworth). ES cells were maintained as described previously (Penny et al., 1996). XX fibroblast cell line C127 was maintained as described previously (Jeppesen and Turner, 1993).

Sequence Analysis

Sequence of 9 kb upstream of the previously reported promoter was obtained from a λ clone isolated from a C57Bl/10 genomic DNA

library and has been deposited in GenBank. Sequence composition analysis was performed using WINDOW (window size = 100, shift = 3) and STATPLOT on the Wisconsin package (Devereux et al., 1984).

RNA Hybridization Analysis

Total RNA was prepared from up to 1×10^8 cells in culture or from 1–200 mg of tissue using the RNeasy midi kit (Qiagen). Slot blots were prepared and hybridizations carried out as described previously (Sheardown et al., 1997a) using 10 μg RNA per slot and 2 ng *Xist* cosmid as a control. *Xist* signal was assessed by quantitative phosphorimaging (Molecular Dynamics; Imagequant). Duplicate samples were subtracted for background, then normalized to 28s rRNA and to cosmid signal. Probes used were as follows: mx1, 628 bp (corresponding to position +1547 to +2205); mx2, 965 bp (−13 to +952); mx3, 1042 bp (−1101 to −59); mx4, 474 bp (−2703 to −2229); mx5, 599 bp (−5470 to −4871); mx6, 520 bp (−7483 to −6963). All distances are in relation to P_1. *Xist* RNA stability assay was carried out as described previously (Sheardown et al., 1997a). Total RNA from aliquots of 1×10^6 cells was assayed in duplicate for each probe. Probes used to detect *Xist* signal were mx2, which detects transcripts from P_1 only in somatic cells, and w7d (Brockdorff et al., 1992), which detects transcripts from $P_1 + P_2$.

RT-PCR Analysis

Preparation of cDNA and RT-PCR analysis was carried out as described previously (Kay et al., 1993). Standard conditions for PCR were as follows: denaturation at 94°C, 3 min; 30–35 cycles of 94°C/55°C/72°C for 1 min each; extension at 72°C for 10 min. Primer pairs generated products spanning regions as follows: CJ1/CJ2: +859 to +1295 (436 bp); CJ3/CJ4: −1247 to −1470 (223 bp); CJ5/CJ6: −5470 to −4871 (599 bp); CJ7/CJ8: −8534 to −8874 (340 bp); CJ9/CJ10: −6029 to −6393 (364 bp); CJ11/CJ12: −6241 to −6601 (360 bp); CJ12/CJ13: −6241 to −6735 (494 bp); CJ14/CJ15: −7463 to −7817 (354 bp).

Nuclease Protection Assay

Xist sequences generated by PCR were cloned into pBluescript (Stratagene). Single-stranded antisense RNA probes of known length were generated from linear plasmid template by transcription from the T3 or T7 promoter using the MaxiScript kit (Ambion). Each probe was labeled with 5 μl ^{32}P UTP, 800 Ci/mmol; 20 mCi/ml and 5 μM cold UTP. Unincorporated nucleotides were removed by excision of full-length probe from a 5% polyacrylamide gel. Probe was recovered by incubating the gel slice in gel elution buffer (Ambion) overnight at 37°C followed by ethanol preciptation. Ten micrograms of RNA was hybridized against 1 fmol labeled probe in S1 hybridization buffer (Ambion) overnight at 42°C, then digested in a total volume of 40 μl with 140 U mung bean nuclease (GIBCO) for 1.5 hr at 37°C or 200 U S1 nuclease (GIBCO) for 30 min at 37°C. Protected products were recovered by ammonium acetate and ethanol precipitation with 10 μg carrier RNA and resolved on a 5% acrylamide denaturing gel alongside an appropriate sequencing ladder.

RNA FISH

Preparation of slides and labeling of double-stranded DNA probes and RNA FISH were carried out as described in Sheardown et al. (1997a). The following *Xist* probes were used to detect nuclear RNA: mx7, a 7 kb exon1 clone (pGPT16, Sheardown et al., 1997a); and mx8, a SacI/SacII fragment spanning −35 bp to approximately −5 kb relative to P_1. mx7 was labeled with biotin and detected with TRITC; mx8 was labeled with digoxigenin and detected with FITC. Strand-specific probes were prepared by linear PCR amplification. Gel-purified PCR product (50 ng) was amplified in the presence of either a sense or an antisense primer (1 μM final concentration). Reactions were performed in 50 μl with final concentrations as follows: 1× PCR buffer (GIBCO BRL); 1.5 mM $MgCl_2$; 2 mM each of dATP, dCTP, and dGTP; 1.9 mM dTTP; 0.1 mM digoxigenin(DIG)-11-dUTP (Boehringer Mannheim) or biotin(BIO)-16-dUTP (Boehringer Mannheim); and 5 U Taq polymerase (GIBCO BRL). Labeled product was generated by 200 cycles of 94°C/30 s; 55°C/30 s; 72°C/2 min. Single-stranded PCR product was detected by visualization on a 1% agarose gel. Fifty nanograms of labeled probe was used per slide.

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References

Ariel, M., Robinson, E., McCarrey, J.R., and Cedar, H. (1995). Gamete-specific methylation correlates with imprinting of the murine *Xist* gene. Nature Genet. *9*, 312–315.

Atwater, J.A., Wisdom, R., and Verma, I.M. (1990). Regulated mRNA stability. Annu. Rev. Genet. *24*, 519–541.

Ayoubi, T.A.Y., and Van de Ven, W.J.M. (1996). Regulation of gene expression by alternative promoters. FASEB J. *10*, 453–460.

Borsani, G., Tonlorenzi, R., Simmler, M.C., Dandolo, L., Arnaud, D., Capra, V., Grompe, M., Pizzuti, A., Muzny, D., Lawrence, C., et al. (1991). Characterization of a murine gene expressed from the inactive X chromosome. Nature *351*, 325–329.

Brockdorff, N., Ashworth, A., Kay, G.F., Cooper, P., Smith, S., McCabe, V.M., Norris, D.P., Penny, G.D., Patel, D., and Rastan, S. (1991). Conservation of position and exclusive expression of mouse *Xist* from the inactive X chromosome. Nature *351*, 329–331.

Brockdorff, N., Ashworth, A., Kay, G.F., McCabe, V.M., Norris, D.P., Cooper, P.J., Swift, S., and Rastan, S. (1992). The product of the mouse *Xist* gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. Cell *71*, 515–526.

Brown, C.J., Ballabio, A., Rupert, J.L., Lafreniere, R.G., Grompe, M., Tonlorenzi, R., and Willard, H.F. (1991a). A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. Nature *349*, 38–44.

Brown, C.J., Lafreniere, R.G., Powers, V.E., Sebastio, G., Ballabio, A., Pettigrew, A.L., Ledbetter, D.H., Levy, E., Craig, I.W., and Willard, H.F. (1991b). Localization of the X inactivation centre on the human X chromosome in Xq13. Nature *349*, 82–84.

Brown, C.J., Hendrich, B.D., Rupert, J.L., Lafreniere, R.G., Xing, Y., Lawrence, J., and Willard, H.F. (1992). The human *XIST* gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. Cell *71*, 527–542.

Cianetti, L., Di Cristofaro, A., Zappavigna, V., Bottero, L., Boccoli, G., Testa, U., Russo, G., Boncinelle, E., and Peschle, C. (1990). Molecular mechanisms underlying the expression of the human *HOX-5.1* gene. Nucleic Acids Res. *18*, 4361–4368.

Clemson, C.M., McNeil, J.A., Willard, H.F., and Lawrence, J.B. (1996). *XIST* RNA paints the inactive X chromosome at interphase: evidence for a novel RNA involved in nuclear/chromosome structure. J. Cell Biol. *132*, 259–275.

Clerc, P., and Avner, P. (1998). Role of the region 3′ to *Xist* exon 6 in the counting process of X chromosome inactivation. Nature Genet. *19*, 249–253.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. *12*, 387–395.

Heard, E., Clerc, P., and Avner, P. (1997). X-chromosome inactivation in mammals. Annu. Rev. Genet. *31*, 571–610.

Hendrich, B.D., Brown, C.J., and Willard, H.F. (1993). Evolutionary conservation of possible functional domains of the human and murine *XIST* genes. Hum. Mol. Genet. *2*, 663–672.

Hendrich, B.D., Plenge, R., and Willard, H.F. (1997). Identification and characterisation of the human *XIST* promoter: implications for models of X chromosome inactivation. Nucleic Acids Res. *25*, 2661–2671.

Herzing, L.B.K., Romer, J.T., Horn, J.M., and Ashworth, A. (1997).

Xist has properties of the X-chromosome inactivation centre. Nature *386*, 272–275.

Jeppesen, P., and Turner, B.M. (1993). The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. Cell *74*, 281–289.

Kay, G.F., Penny, G.D., Patel, D., Ashworth, A., Brockdorff, N., and Rastan, S. (1993). Expression of *Xist* during mouse development suggests a role in the initiation of X chromosome inactivation. Cell *72*, 171–182.

Lee, J.T., Strauss, W.M., Dausman, J.A., and Jaenisch, R. (1996). A 450 kb transgene displays properties of the mammalian X-inactivation center. Cell *86*, 83–94.

Lee, J.T., and Jaenisch, R. (1997). Long range cis effects of ectopic X inactivation centres on a mouse autosome. Nature *386*, 275–279.

Leighton, P.A., Ingram, R.S., Eggenschweiler, J., Efstratiadis, A., and Tilghman, S.M. (1995). Disruption of imprinting caused by deletion of the *H19* region in mice. Nature *375*, 34–39.

Marahrens, Y., Panning, B., Dausman, J., Strauss, W., and Jaenisch, R. (1997). *Xist* deficient mice are defective in dosage compensation but not spermatogenesis. Genes Dev. *11*, 156–166.

Marahrens, Y., Loring, J., and Jaenisch, R. (1998). Role of the *Xist* gene in X chromosome choosing. Cell *92*, 657–664.

Marcu, K.B., Bossone, S.A., and Patel, A.J. (1992). *myc* function and regulation. Annu. Rev. Biochem. *61*, 809–860.

McMahon, A., Fosten, M., and Monk, M. (1983). X chromosome inactivation mosaicism in the three germ layers and the germ line of the mouse embryo. J. Embryol. Exp. Morphol. *46*, 53–64.

Monk, M., and Harper, M.I. (1979). Sequential X chromosome inactivation coupled with cellular differentiation in early mouse embryos. Nature *281*, 311–313.

Norris, D.P., Patel, D., Kay, G.F., Penny, G.D., Brockdorff, N., Sheardown, S.A., and Rastan, S. (1994). Evidence that random and imprinted *Xist* expression is controlled by preemptive methylation. Cell *77*, 41–51.

Panning, B., and Jaenisch, R. (1996). DNA hypomethylation can activate *Xist* expression and silence X-linked genes. Genes Dev. *10*, 1991–2002.

Panning, B., Dausman, J., and Jaenisch, R. (1997). X chromosome inactivation is mediated by *Xist* RNA stabilization. Cell *90*, 907–916.

Penny, G.D., Kay, G.F., Sheardown, S.A., Rastan, S., and Brockdorff, N. (1996). Requirement for *Xist* in X chromosome inactivation. Nature *379*, 131–137.

Rastan, S. (1983). Non-random X chromosome inactivation in mouse X autosome translocations: location of the inactivation center. J. Embryol. Exp. Morphol. *78*, 1–22.

Rastan, S., and Robertson, E.J. (1985). X chromosome deletions in embryo-derived (EK) cell lines associated with lack of X chromosome inactivation. J. Embryol. Exp. Morphol. *90*, 379–388.

Rastan, S., and Brown, S.D. (1990). The search for the mouse X-chromosome inactivation centre. Genet. Res. *56*, 99–106.

Sheardown, S.A., Duthie, S.M., Johnston, C.M., Newall, A.E.T., Formstone, E.J., Arkell, R.M., Nesterova, T.B., Alghisi, G.-C., Rastan, S., and Brockdorff, N. (1997a). Stabilization of *Xist* RNA mediates initiation of X chromosome inactivation. Cell *91*, 99–107.

Sheardown, S.A., Newall, A.E.T., Norris, D.P., Rastan, S., and Brockdorff, N. (1997b). Regulatory elements in the minimal promoter region of the mouse *Xist* gene. Gene *203*, 159–168.

Takagi, N., and Sasaki, M. (1975). Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse. Nature *256*, 640–642.

Zuccotti, M., and Monk, M. (1995). Methylation of the mouse *Xist* gene in sperm and eggs correlates with imprinted *Xist* expression and paternal X-inactivation. Nature Genet. *9*, 316–332.

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